Podocalyxin inhibits human embryo implantation in vitro and luminal podocalyxin in putative receptive endometrium is associated with implantation failure in fertility treatment

Sophea Heng, Ph.D.,[a] Nirukshi Samarajeewa, Ph.D.,[a] Asma Aberkane, Ph.D.,[b] Wafaa Essahib, M.B.S.,[b] Hilde Van de Velde, Ph.D.,[b] Maxine Scelwyn, M.D.,[c] M. Louise Hull, M.D., Ph.D.,[d] Beverley Vollenhoven, M.D., Ph.D.,[e,f] Luk J. Rombauts, M.D., Ph.D.,[e,f] and Guiying Nie, Ph.D.[a,g]

[a] School of Health and Biomedical Sciences, Royal Melbourne Institute of Technology University, Victoria, Australia; [b] Research Group of Reproduction and Immunology, Vrije Universiteit Brussel, Brussels, Belgium; [c] Anatpath, Gardenvale, Victoria, Australia; [d] Robinson Research Institute, University of Adelaide, Adelaide, South Australia, Australia; [e] Department of Obstetrics and Gynaecology, Monash University, Clayton, Victoria, Australia; [f] Womens and Newborn Programme, Monash Health, Clayton, Victoria, Australia; and [g] Hudson Institute of Medical Research, Clayton, Victoria, Australia

Objective: To study whether endometrial epithelial podocalyxin (PCX) inhibits implantation of human embryos in vitro and in patients undergoing in vitro fertilization (IVF).
Design: We have recently identified PCX as a key negative regulator of endometrial epithelial receptivity. Podocalyxin is expressed in all epithelial cells in the nonreceptive endometrium, but is selectively downregulated in the luminal epithelium (LE) for receptivity. In the current study, we first investigated whether high levels of PCX in Ishikawa monolayer inhibit attachment and/or penetration of human blastocysts in in vitro models. We then examined PCX by immunohistochemistry in putative receptive endometrial tissues biopsied from 81 IVF patients who underwent frozen embryo transfer in the next natural cycle and retrospectively analyzed the association between PCX staining in LE and clinical pregnancy as a proxy of successful implantation.
Setting: RMIT University, Australia; Vrije Universiteit Brussel, Belgium.
Patient(s): In vitro fertilization patients undergoing frozen/thawed embryo transfer.
Intervention(s): N/A.
Main Outcome Measure(s): Endometrial epithelial PCX inhibits implantation of human embryos in vitro and in IVF patients.
Result(s): High levels of PCX in Ishikawa monolayer significantly inhibited blastocyst attachment and penetration. Among the 81 putative receptive tissues, 73% were negative, but 27% were heterogeneously positive for PCX in LE. The clinical pregnancy rate was 53% in those with a PCX-negative LE but only 18% in those with a PCX-positive LE. If LE was positive for PCX, the odds ratio of no clinical pregnancy was 4.95 (95% Confidence interval, 1.48–14.63).

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Reprint requests: Guiying Nie, Ph.D., Implantation and Pregnancy Research Laboratory, School of Health and Biomedical Sciences, Royal Melbourne Institute of Technology University, Bundoora West Campus, Victoria 3083, Australia (E-mail: guiying.nie@rmit.edu.au).

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Conclusion(s): Podocalyxin inhibits embryo implantation. Assessment of PCX may aid the evaluation and optimization of endometrial receptivity in fertility treatment. (Fertil Steril® 2021;116:1391–401. ©2021 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Endometrial receptivity, embryo implantation, epithelial receptivity, IVF, Podocalyxin,

Embryo implantation requires synchronized interactions between a well-developed blastocyst and a receptive endometrium (1–4). In the human, embryo–endometrial interaction begins when a blastocyst opposes itself to the endometrial luminal epithelium (LE), the embryo then firmly attaches to and penetrates through this epithelial layer, eventually imbedding itself within the subepithelial stroma (3, 5, 6). For implantation to succeed, the trophectoderm (TE) of the blastocyst and the LE of the endometrium, which are normally mutually repulsive, must come together to initiate adhesion. However, to date, knowledge of luminal epithelial remodeling for human endometrial receptivity is very limited. This lack of knowledge impedes the improvement of fertility treatment, where seemingly high-quality embryos fail to implant (7–10).

In general, the transition of endometrial epithelium from a nonreceptive to a receptive state involves major cellular changes, including loss of tight junction integrity, cell–cell contacts, and apical–basal cell polarity, which share some phenotypes of epithelial–mesenchymal transition (11–14). It is well known that endometrial conversion to a receptive state is driven primarily by progesterone, and several factors, including adhesion molecules and cytokines, have been identified to play crucial roles (6, 15–17). However, it is not well understood whether all epithelial cells within the human endometrium change similarly for receptivity or the LE remodels distinctively.

We have recently discovered that membrane protein podocalyxin (PCX) is a critical negative regulator of human endometrial epithelial receptivity (18). Podocalyxin is strongly expressed on the apical surfaces of all luminal and glandular epithelial cells, as well as vascular endothelial cells, in the nonreceptive endometrium. However, in the midsecretory phase when receptivity is established, PCX is downregulated selectively and specifically in the LE (18). We have further shown that this downregulation is likely mediated by progesterone because primary human endometrial epithelial cells in culture significantly reduce PCX mRNA and protein when treated with progesterone (18).

Podocalyxin is a single-pass transmembrane protein belonging to the CD34 family of sialomucins, it is also known as PODXL, PCLP1, gp135, MEP21, and thrombomucin (19). Podocalyxin is known to be expressed in podocytes, hematopoietic progenitor cells, vascular endothelial cells, and some epithelial cancers (20–22). Podocalyxin in podocytes acts as an antiadhesive protein and is essential for the formation and maintenance of podocyte foot processes in an open state (22). In addition, the antiadhesive function of PCX has been implicated in a number of poorly differentiated epithelial cancers (22–24). Dysregulation of PCX affects cellular functions through its interaction with adaptor proteins implicated in protein trafficking, ion transport, signaling (25–27), and actin binding protein, ezrin (28, 29).

We have thus investigated the functional importance of PCX in embryo implantation (18). We stably overexpressed PCX in Ishikawa cells, a commonly used receptive human endometrial epithelial cell line, and showed that PCX profoundly increased the polarity of Ishikawa cells, a phenotype associated with nonreceptivity. Functionally, PCX overexpression in Ishikawa cells significantly inhibited not only attachment but also invasion of trophoblast spheroids as embryo surrogates (18). We have thus proposed that PCX acts as a negative regulator of endometrial epithelial receptivity and that its specific downregulation in LE would selectively render this layer of cells, which are to directly interact with the implanting embryo, amenable to attachment and invasion (18).

To further prove the aforementioned proposition, the current study aimed to investigate whether PCX inhibits implantation of actual human embryos in in vitro models, utilizing our previously established Ishikawa cell line stably overexpressing PCX. In addition, the study endeavored to determine whether the presence of PCX in LE in vitro fertilization (IVF) patients around the time of embryo transfer is associated with implantation failure.

MATERIALS AND METHODS

Ethical Statement Regarding the Use of Human Embryos

All experiments involving human embryos were performed at the Centre for Reproductive Medicine in CRG, UZ Brussels, Belgium. The use of cryopreserved human embryos was approved by the Institute Ethical Committee (BUN143201316309) and the Federal Committee for Scientific Research on Human Embryos in vitro (AdV045). With written informed consent, embryos used for this study were donated for research after the legally determined cryopreservation period of 5 years (30).

Culture of Control and PCX-Overexpressing Ishikawa Cells

Control and PCX-overexpressing (PCX-OE) Ishikawa cells, previously established in our laboratory (18), were cultured in complete medium containing Minimum Essential Media (Thermo Fisher Scientific, USA) supplemented with 10% v/v fetal bovine serum (Bovogen Biologicals, Australia), 1% v/v

antibiotic–antimycotic, 1% v/v L-glutamine (Thermo Fisher Scientific), and 2% v/v G418 (Sigma Aldrich, USA).

Assessment of Human Embryo Attachment to Ishikawa Monolayer

Control or PCX-OE Ishikawa cells were cultured at 37 °C under 5% CO_2 in 96-well flat-bottom plates to form a monolayer. Prior to coculture with human embryos, Ishikawa cells were replenished with 200 μL fresh complete medium. Good-quality, vitrified, 5-day postfertilization (dpf) blastocysts, full and expanding with A or B scoring for both inner cell mass and TE according to the Gardner and Schoolcraft criteria (31), were warmed using the Vitrification Thaw Kit (Vit Kit-Thaw, Irvine Scientific, USA) following the manufacturer's protocol and transferred into 25 μL droplets of Origio blastocyst medium (Origio, Denmark) for recovery at 37 °C with 5% O_2, 6% CO_2, and 89% N_2. To assist with embryo hatching, a hole was made in the zona pellucida (ZP) using a laser. Based on morphological scoring, good-quality 6dpf embryos hatched from the ZP were used for further experiments. Each embryo was removed from its culture droplet, rinsed with Ishikawa complete medium, transferred to the top of control or PCX-OE monolayers, and cocultured for 15 or 24 hours at 37 °C under 5% CO_2 humidified incubator. Embryo attachment to Ishikawa monolayer was assessed under a stereological light microscope (Nikon, Japan) where 100 μL medium was gently pipetted up and down 3 to 4 times using a 200 μL tip. Free-floating embryos were considered unattached. The attachment rate was calculated as the percentage of attached/transferred embryos. A total of 11 embryos were assessed for each condition in three separate experiments.

Assessment of Human Embryo Traversing Through Ishikawa Monolayer

A monolayer of control and PCX-OE Ishikawa cells was prepared on a layer of matrix on glass coverslip slides containing 8-well chambers as previously described (18). Ishikawa cells were cultured on top of this matrix in complete medium to form a monolayer overnight at 37 °C under 5% CO_2 incubator. The following day, cells were replenished with fresh medium containing Vybrant cell-labeling solution DiI (Thermo Fisher Scientific, 5 μL per 1 mL of medium) and incubated for another 24 hours. Before coculture with human embryos, each well was washed with phosphate-buffered saline, replenished with fresh medium and equilibrated for 4 hours at 37 °C under 5% CO_2 incubator.

Good-quality vitrified 3dpf embryos at compaction C1 and C2 stages (32) were warmed using the Vitrification Thaw Kit (Vit Kit-Thaw, Irvine Scientific) following the manufacturer's protocol and transferred into 25 μL droplets of Origio blastocyst medium for recovery at 37 °C with 5% O_2, 6% CO_2, and 89% N_2. A large hole was made in the ZP of each 4dpf embryo using a laser, and the embryos were left to recover overnight. The next day, good-quality 5dpf blastocysts were transferred into culture droplets containing Vybrant cell-labeling solution DiO (Thermo Fisher Scientific, 10 μL per 1 mL of medium) and incubated for 24 hours at 37 °C with 5% O_2, 6% CO_2, and 89% N_2. Good-quality (morphological scoring) 6dpf embryos hatched from the ZP were used for the invasion assay. Each embryo was removed from the culture droplet, rinsed with Ishikawa complete medium, transferred to the top of control or PCX-OE monolayer, and cocultured for 24 hours at 37 °C under 5% CO_2 incubator. After coculture, each chamber was imaged using confocal microscopy (Zeiss, Germany).

Confocal Analysis of Human Embryo Penetration Through Ishikawa Monolayer

Surface mapping for human embryos cocultured with Ishikawa monolayers (control or PCX-OE) was performed using the Imaris software (version 9.2.1, Bitplane, AG). The extent of invasion was quantified by analyzing the volume of the embryo that penetrated through the monolayer and was present beneath the Ishikawa monolayer.

Analysis of PCX in Endometrial Tissues from IVF patients and Its Association with Clinical Pregnancy Outcomes

A cohort of archived endometrial tissues (n = 81) collected from the endometrial scratch procedure during fertility treatment between 2012 and 2017 at Monash IVF in Australia were retrieved and analyzed for PCX by immunohistochemistry. All biopsies were taken in the midsecretory phase of the menstrual cycle immediately preceding a transfer cycle. Cycle stage was analyzed and confirmed using histological dating criteria (33) by Anatpath Services (Gardenvale, VIC, Australia) after the tissues were fixed in formalin and imbedded in paraffin. In the immediate next cycle, at least one good-quality (grade C or above) frozen-thawed embryo at the cleavage or blastocyst stage was replaced in a natural cycle on day 3 or day 5 after ovulation as detected by serial luteinizing hormone measurements in serum. Baseline patient demographics and clinical pregnancy outcomes were additionally retrieved. A clinical pregnancy was defined based on the Australian and New Zealand Assisted Reproductive Database definition, which includes pregnancies that are known to be ongoing at 20 weeks or are evidenced by ultrasound of an intrauterine sac and/or fetal heart. Ethics approval for this study was obtained from Monash Health (#RES-16-0000418L).

Podocalyxin was immunostained as previously described (18). In brief, 5 μm freshly cut sections were deparaffinized, rehydrated and microwaved in 0.01 M citrate buffer at pH 6.0. Sections were blocked for 20 min at room temperature (RT) with 15% v/v horse serum in high salt tris-buffered saline (0.3 M NaCl, 0.05 M Tris base, pH 7.6) containing 0.1% v/v Tween20 (Sigma) and then incubated for 1 hour at 37 °C with primary PCX antibody (2 μg/mL, SC-23904, Santa Cruz, USA) in 10% v/v fetal bovine serum in high salt tris-buffered saline containing 0.1% v/v Tween20. The primary antibody was replaced with mouse immunoglobulin G (Dako, USA) in the negative control. Sections were then incubated for 30 min at RT first with biotinylated horse anti-mouse immunoglobulin G (#BA-2000, Vector Laboratories, Inc., USA) and then with StreptABC/HRP (Dako), and signals were visualized with diaminobenzidine (Dako). Nuclei were

stained with hematoxylin (blue), and images were analyzed using bright-field microscopy (Olympus Optical, Tokyo, Japan). Podocalyxin staining was assessed by two independent observers who were blinded to the clinical pregnancy outcomes, and a PCX-positive LE was defined when more than 50% of LE was stained. The association between a PCX-positive LE and clinical pregnancy (as a proxy of successful implantation) was analyzed by Fisher's exact test.

To investigate the impact of the slide age on PCX immunostaining, serial sections were cut from five tissue blocks, dried at 37 °C overnight, and stored at RT. Podocalyxin immunostaining was performed on fresh and stored sections at a monthly interval by the same person with the same protocol.

Statistical Analysis

GraphPad Prism version 9.0.1 (GraphPad Software, San Diego, CA) was used for statistical analysis. Analysis of variance and Mann-Whitney *U* test were applied for the in vitro studies, and Fisher's exact test and odds ratio (OR) calculation determined the association between PCX staining in LE and clinical pregnancy outcomes. Significance was defined as $P \leq .05$ and $P \leq .01$.

RESULTS

PCX in Ishikawa Cell Monolayer Inhibits Attachment of Human Blastocysts

In our previously reported in vitro implantation model (18, 34), we cocultured trophoblast spheroids (as surrogates of human embryos) on a monolayer of Ishikawa cells, a commonly used receptive human endometrial epithelial cell line, as a mimic of human endometrial epithelial surface. To investigate the impact of PCX on endometrial epithelial receptivity, we stably overexpressed human PCX in Ishikawa cells to imitate the nonreceptive state (18) and showed that PCX-OE significantly inhibited the ability of trophoblast spheroids to attach to and invade through the Ishikawa monolayer (18). Using these Ishikawa cells, here, we examined whether PCX inhibits attachment of actual human embryos in the in vitro model. Equal numbers of human blastocysts were cocultured on top of control or PCX-OE Ishikawa monolayer, and stable attachment was assessed at 15 hours. On the control Ishikawa monolayer, 64% (7 out of 11) of added blastocysts firmly attached (Fig. 1A). However, on the PCX-OE monolayer, embryo attachment was only 27% (3 out of 11), which was significantly lower than on the control monolayer (Fig. 1A, $P = .025$). The experiment was repeated, but attachment was examined at 24 hours; by then approximately 78% (7 out of 9) of added blastocysts attached on both monolayers (data not shown). These data demonstrated that PCX in the Ishikawa monolayer hinders and reduces the speed of blastocyst attachment, which is consistent with our previous observation with trophoblast spheroids (18). Of note, by 24 hours of coculture, embryo invasion has already occurred, which was investigated next.

PCX in Ishikawa Monolayer Inhibits Penetration of Human Blastocysts

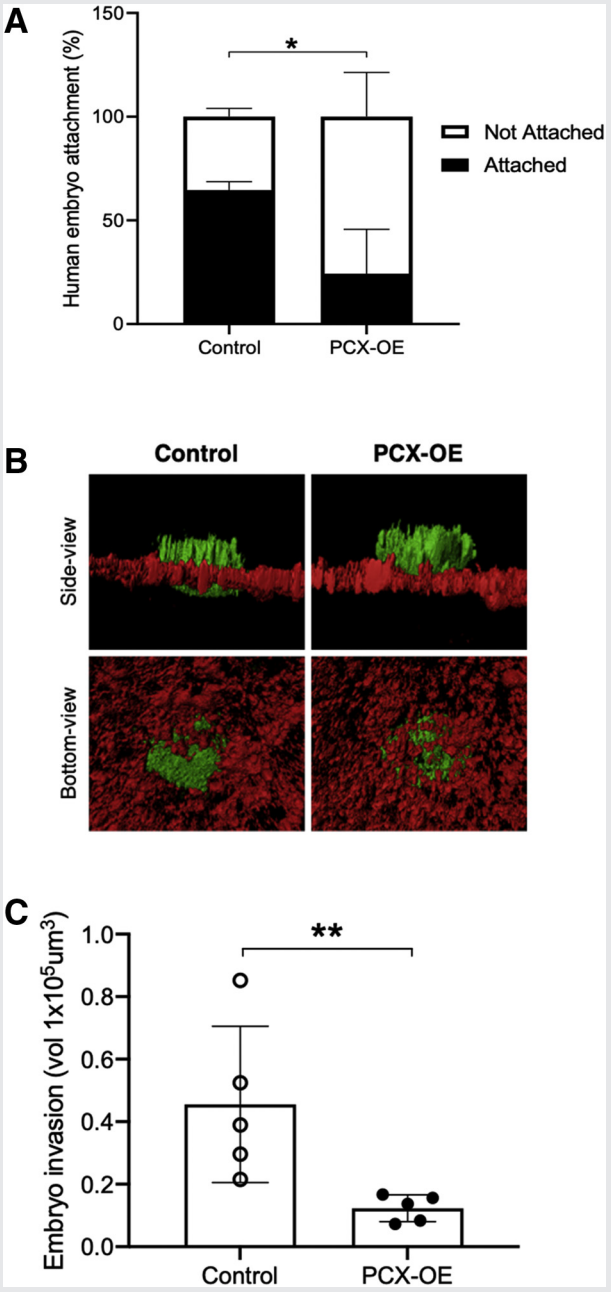
Human implantation requires the embryo to attach to the LE, penetrate the epithelial cell layer, and move to the stroma (3). We then investigated whether PCX in Ishikawa cells affects the ability of human blastocysts to traverse through the monolayer. To assist the analysis, we first labeled blastocysts and Ishikawa cells with different Vybrant cell-labeling DiO and DiI dyes respectively, then cocultured the dye-labeled blastocysts on top of a dye-labeled Ishikawa monolayer for 24 hours, and examined the position of the embryo within the monolayer by confocal z-stack imaging (a total of five embryos per group). Embryos clearly invaded through the control Ishikawa monolayer, whereas penetration was much less through the PCX-OE Ishikawa monolayer (Fig. 1B). To assess the degree of invasion, we quantified the volume of embryos that had penetrated the Ishikawa monolayer and was present underneath the monolayer (Fig. 1C). Embryo volume under the PCX-OE monolayer was significantly lower (by 70%) than that under the control monolayer ($P = .008$). These data provide experimental evidence that PCX in endometrial epithelial cells inhibits human embryo invasion.

A PCX-Positive LE in Putative Receptive Endometrium of IVF Patients Is Associated with Failure of Clinical Pregnancy in the Next Cycle of Frozen Embryo Transfer

Data from the aforementioned in vitro model with human blastocysts, together with our previous studies (18), consistently suggest that PCX is a negative regulator of endometrial epithelial receptivity for embryo implantation. To further prove this notion, we next examined PCX in endometrial tissues obtained from IVF patients and retrospectively analyzed the association with implantation outcomes. Because receptive endometrial tissue cannot be biopsied in the cycle of embryo transfer, tissues obtained in the preceding cycle were examined. We retrieved 81 endometrial tissues biopsied during the endometrial scratch procedure from women undergoing fertility treatment. All biopsies were taken at the time when the endometrium is putatively receptive (midsecretory phase of the menstrual cycle), and the cycle stage was confirmed by experienced pathologists based on histological dating. All patients received transfer of a single or double frozen-thawed good-quality embryos in the next natural cycle.

For these endometrial tissues, we first examined whether their PCX staining pattern is consistent with them being in the receptive phase as deemed by histological dating. We based our analysis on the following PCX expression pattern as reported in our previous study (18) and summarized in the table in Figure 2A as follows: the prereceptive phase is characterized by strong PCX presentation on the apical surface of luminal and glandular epithelium; the receptive phase is signified by marked PCX downregulation in the LE but persistent PCX expression in glandular epithelium; the late-receptive phase is associated with little luminal and

FIGURE 1



Podocalyxin (PCX) inhibits human embryo attachment and invasion. **(A)** Equal numbers of blastocysts were cocultured on top of control or PCX-overexpressing (PCX-OE) Ishikawa monolayer, and the percentage of embryo attachment (number of attached/added) was examined at 15 hours. A total of 11 blastocysts were used on each monolayer in three separate experiments. **(B)** Dye-labeled human blastocysts were cocultured on dye-labeled control or PCX-overexpressing (PCX-OE) Ishikawa monolayer for 24 hours. Representative confocal images of side and bottom views of human embryos (*green*) and Ishikawa monolayers (*red*). **(C)** Quantification of embryo invasion. The volume of embryo present beneath the Ishikawa monolayer was quantified by surface mapping (n = 5). Data are expressed as mean ± SD. *$P<.05$ and **$P<.01$.

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low glandular PCX staining. Endothelial cells show strong PCX staining across the menstrual cycle with no obvious cyclic changes.

Among the 81 tissues examined, very few showed continuous PCX staining uniformly throughout the entire LE like a typical proliferative sample, consistent with them being biopsied in the secretory phase. However, while most showed no luminal PCX staining as expected for a receptive phase tissue, some showed strong, heterogeneous, and long stretches of PCX staining in ≥50% of their LE, and these were defined as luminal PCX-positive. On the basis of these criteria, of the 81 endometrial tissues, 69% (n = 56) had a PCX staining pattern resembling a receptive phase (strong glandular but little luminal staining), but 27% (n = 22) displayed prereceptive characteristics (strong glandular but in addition positive and heterogeneous luminal PCX staining), and the remaining 4% (n = 3) were more like late-receptive (faint glandular and little luminal PCX staining) (Pie chart in Fig. 2A). Representative images of each of these phases are shown in Figure 2B. All these 81 tissues displayed strong PCX staining in endothelial cells throughout the endometrium as expected.

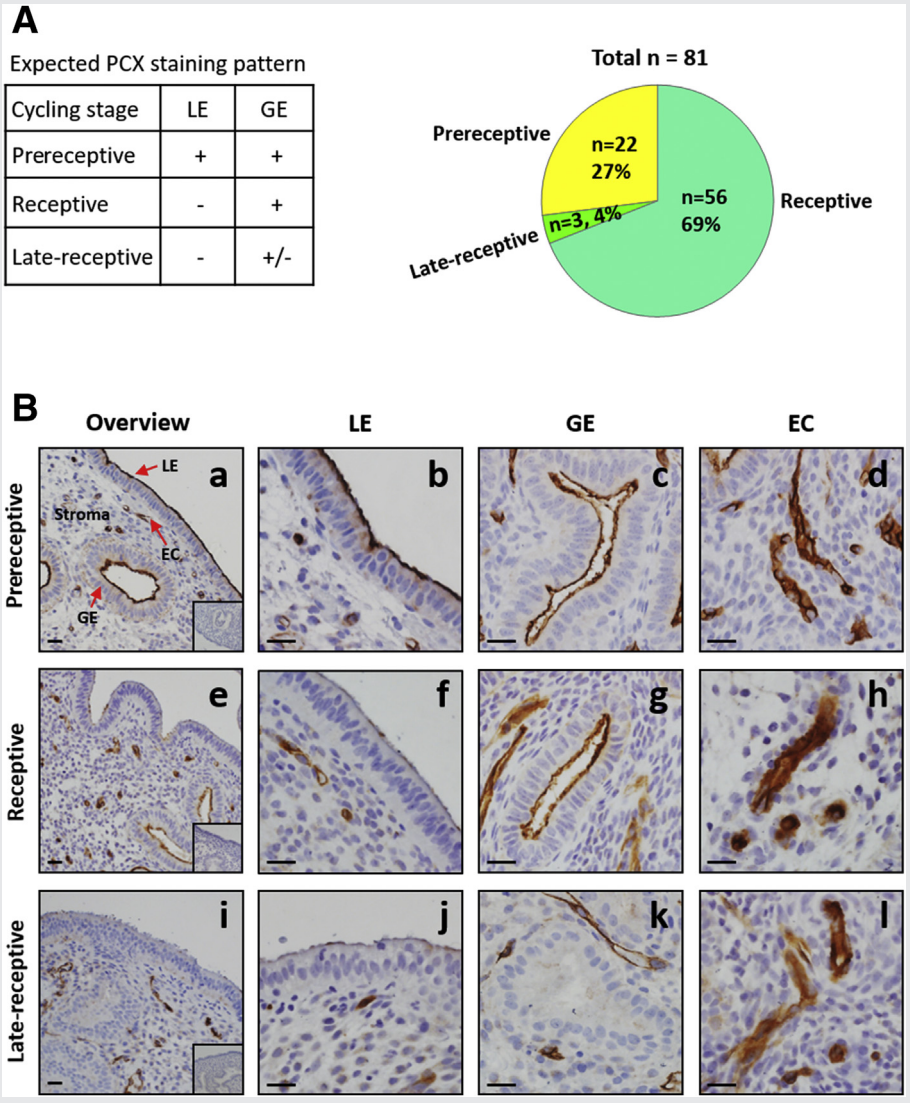
Because only three tissues were predicted as late-receptive but they still presented with a PCX-negative LE, we next clustered these 3 samples together with the receptive tissues (n = 56) as the luminal PCX-negative group (total n = 59, 73%) and compared their implantation outcomes with that of the prereceptive ones as the luminal PCX-positive group (n = 22, 27%) (Pie chart in Fig. 3). As embryo implantation cannot be directly assessed, clinical pregnancy was used as a proxy of successful implantation.

The overall clinical pregnancy rate of the total cohort of n = 81 was 43% (57% failure) (Fig. 3, Table 1). When they were analyzed on the basis of the luminal PCX staining, the clinical pregnancy rate was 53% (47% failure) in those with a PCX-negative LE, but it was only 18% (82% failure) in those with a PCX-positive LE (Fig. 3, Table 1, $P = .006$). If PCX was positive compared with negative in the LE, the OR of no clinical pregnancy was 4.95 (95% Confidence interval [CI], 1.48–14.63) (Table 1).

The analysis was repeated by including only those who had multiple prior failed embryo transfers (≥3 cycles), which encompassed a total of n = 61 patients, who had an overall clinical pregnancy rate of 43% (57% failure) (Table 1). Among these, 43 (70%) had a PCX-negative LE, and their clinical pregnancy rate was 53% (47% failure), whereas the remaining 18 (30%) showed a PCX-positive LE and their clinical pregnancy rate was only 17% (83% failure) (Table 1). Between the two groups, Fisher's exact test $P = .011$, and the OR was 5.75 (95% CI, 1.47–20.30) if the LE was positive for PCX. These results largely resembled those when all samples were included.

In addition, we analyzed only those who received a single embryo transfer (total n = 73, Table 1). Of these, 54 (74%) were PCX-negative, whereas 19 (26%) were PCX-positive in their LE, and their clinical pregnancy rates were 50% (50% failure) and 16% (84% failure) respectively. Again, the two groups differed significantly ($P = .014$) with a OR of 5.33

FIGURE 2



Analysis of podocalyxin (PCX) staining in the human endometrial tissues biopsied from IVF patients in the putative receptive phase. **(A) Table**, PCX staining pattern expected for various stages of the menstrual cycle based on prior studies. GE = glandular epithelium; LE = luminal epithelium. **Pie chart**, classification of the 81 tissues examined into prereceptive, receptive, and late-receptive phases by number (n) and percentage (%), based on their PCX staining pattern. **(B)** Representative images of tissues showing characteristics of each specified phase. Shown are overview (a, e, i) and higher magnification of LE (b, f, j), GE (c, g, k), and endothelial cells (EC; d, h, l). Inserts, negative controls. Scale bars, 20 μm.

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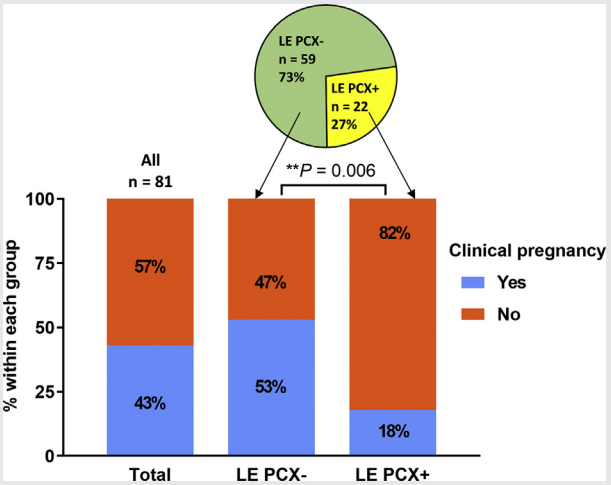
(95% CI, 1.48–18.47). These results largely echoed those when all embryo transfers were included. Furthermore, when the analysis included only those who received a single embryo transfer and had multiple prior failed cycles (≥ 3 cycles, total n = 54, Table 1), similar results were observed (Table 1), with 70% showing a PCX-negative epithelium and 50% clinical pregnancy rate and the other 30% presenting a PCX-positive epithelium and a 19% clinical pregnancy rate (Table 1; $P = .039$; OR, 4.33). Maternal age did not differ among any of these groups.

These data showed a strong association between a PCX-positive LE and failure of clinical pregnancy in IVF patients, consistent with luminal PCX hindering embryo implantation. In addition, these results suggest a potential utility of luminal PCX as a biomarker in classifying the receptive status of the endometrial surface.

Immunostaining for PCX Requires Tissue Slides Stored Less Than 3 Months After Sectioning

During our studies, the intensity of PCX immunostaining was noticed to be weaker on prior sectioned tissue slides, consistent with previous reports for a number of proteins (35–39). To determine the time threshold under which tissue sections

FIGURE 3



Luminal podocalyxin (PCX) staining status in the putative receptive endometrium of IVF patients and their clinical pregnancy outcomes in the next frozen embryo transfer cycle. **Pie chart**, the distribution of the 81 tissues between groups with a PCX-negative (PCX−) and PCX-positive (PCX+) LE by number (n) and percentage (%). **Stacked bar chart**, clinical pregnancy rate in the whole cohort (Total) and in the LE PCX− and PCX+ groups respectively; $**P \leq .01$, Fisher's exact test.

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can be stored for optimal PCX immunostaining, serial sections were cut from five tissues and stored at RT. Podocalyxin immunostaining was performed on freshly cut sections and then on stored sections at a monthly interval by the same person with the same protocol. Overall, similar PCX staining was observed for the first three months, after which a clear reduction in signal was apparent. Representative images of tissues with a PCX-positive and PCX-negative LE are shown in Supplemental Figure 1 (available online). These results suggest that assessment of PCX by immunostaining requires tissue slides that are stored for less than 3 months after sectioning.

DISCUSSION

Successful embryo implantation requires a receptive endometrium in addition to an implantation-competent blastocyst, and the two components need to be developed in synchrony. In this study, using in vitro implantation models and human endometrial biopsies of IVF patients, we investigated endometrial receptivity in relation to PCX, a potentially important negative regulator of human endometrial epithelial receptivity that we have recently identified (18). Data from the in vitro models showed that PCX in endometrial epithelial cells inhibits the attachment as well as the penetration of human blastocysts. Analyzing endometrial tissues biopsied from IVF patients, we further demonstrated that a putative receptive endometrium with a PCX-positive LE is significantly associated with implantation failure in the next natural cycle of frozen embryo transfer. These data collectively provide compelling evidence supporting the notion that PCX is a major factor associated with a nonreceptive epithelium and that PCX downregulation in the LE signifies the transition of endometrial surface to an implantation-permissive state. In addition, our results revealed the potential clinical utility of luminal PCX to evaluate the status of endometrial surface receptivity.

The initial process of embryo implantation involves close interactions between two layers of epithelial cells, the TE and endometrial LE, which would conventionally be

TABLE 1

Comparison of clinical pregnancy rates between patients who showed a PCX-negative (PCX$^-$) and those who showed a PCX-positive (PCX$^+$) luminal epithelium.

				Clinicnal pregnancy (CP)			
						Comparison: PCX− vs PCX+ groups	
Embryo transfer	Mean age (years)	PCX staining in LE n (%)		Yes n (%)	No n (%)	Fisher's exact test *P* value	Odds ratio of no CP if PCX+ in LE (95% CI)
All transfers	38.29	Total	81 (100%)	35 (43%)	46 (57%)		
	38.01	PCX−	59 (73%)	31 (53%)	28 (47%)	.006[b]	
	39.04	PCX+	22 (27%)	4 (18%)	18 (82%)		4.95 (1.48–14.63)
All transfers, cycles ≥3	38.30	Total	61 (100%)	26 (43%)	35 (57%)		
	38.18	PCX−	43 (70%)	23 (53%)	20 (47%)	.011[a]	
	38.60	PCX+	18 (30%)	3 (17%)	15 (83%)		5.75 (1.47–20.30)
Single embryo transfers	37.92	Total	73 (100%)	30 (41%)	43 (59%)		
	37.73	PCX−	54 (74%)	27 (50%)	27 (50%)	.014 [a]	
	38.46	PCX+	19 (26%)	3 (16%)	16 (84%)		5.33 (1.48–18.47)
Single embryo transfers, cycles ≥3	37.89	Total	54 (100%)	22 (41%)	32 (59%)		
	37.81	PCX−	38 (70%)	19 (50%)	19 (50%)	.039[a]	
	38.07	PCX+	16 (30%)	3 (19%)	13 (81%)		4.33 (1.01–15.70)

Note: CI = confidence interval; LE = luminal epithelium; PCX = podocalyxin.
[a] $P \leq .05$
[b] $P \leq .01$

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mutually exclusive to one another. Appropriate preparation of the LE for receptivity is thus critical for the initiation of embryo implantation. However, when endometrial tissues are lysed for transcriptomic or proteomic analysis, the unique contribution of LE for receptivity is masked and commonly overlooked. Although it has long been recognized that epithelial receptivity requires plasma membrane remodeling, polarity reduction, and other changes akin to epithelial–mesenchymal transition (12, 14, 40), distinctive features of LE remodeling for receptivity are not well characterized in human.

Our recent studies on PCX have shed significant light on the discrete regulation of LE for endometrial receptivity (18). A key finding is that PCX is expressed at the apical surface of all epithelial and endothelial cells in the human endometrium but is selectively downregulated in LE at receptivity (18). We further showed that PCX increases Ishikawa cell polarity, affecting actin filament organization and membrane localization of polarity marker scribble, changing the cell characteristics toward a nonreceptive state (18). Importantly, we demonstrated that these PCX-induced characteristics render Ishikawa cells functionally incompetent for attachment and penetration of trophoblast spheroids (18). In the current study, we expanded the in vitro models to using actual human blastocysts and further demonstrated that PCX inhibits the attachment and penetration of human embryos. Taken together, these studies provide clear evidence corroborating the proposition that downregulation of PCX in LE is essential for endometrial receptivity. Previous gene deletion studies in endothelial cells show that loss of PCX alters their adhesion to extracellular matrix and changes their expression of junction proteins, resulting in vascular leakage (41, 42). Because embryo implantation would require the LE to be "leaky" with loose cell–cell junctions, future studies are warranted to investigate whether PCX additionally affects the expression and localization of junction proteins in endometrial epithelial cells.

To further prove the aforementioned theory, we examined endometrial tissues biopsied in the putative receptive phase from IVF patients who received frozen-thawed embryos at the similar cycling stage in their next natural cycle. Our data showed that patients with a PCX-positive LE at around time of embryo transfer are far more likely to fail implantation than those with a PCX-negative LE. Because it is impossible to monitor embryo implantation precisely in the human, we resorted to clinical pregnancy as a surrogate outcome. Because receptive endometrium cannot be biopsied in the cycle of embryo transfer, we analyzed endometrial tissues obtained from a preceding cycle. To reduce confounders, we only included patients who underwent frozen embryo transfer in a natural cycle, with no cycle gaps between the sampling and transfer cycles. Despite these limitations, our results are consistent with the in vitro data and firmly support the concept that PCX needs to be downregulated in the LE for successful implantation.

Based on the PCX pattern, our studies indicate that approximately 26%–30% of endometrial tissues biopsied in the putative receptive phase are in the prereceptive phase, which agrees with the results obtained from the microarray-based endometrial receptivity array (ERA) test (43–46). However, PCX is not on the ERA gene list, and the two approaches differ completely. While ERA determines the transcriptomic profile of 238 genes with specific algorithms after lysing the entire endometrial tissue, our analysis examined a single marker on intact tissues leveraging the endometrial architecture. The latter does not require special setups and can be performed in standard pathology operations. It can be speculated that those with a PCX-positive LE may simply need a longer time to reach receptivity. Furthermore, we previously reported that progesterone downregulates PCX in primary epithelial cells (18), which suggests an opportunity of utilizing PCX as a marker to optimize/personalize progesterone dose, type, and even route of administration in luteal support formulations (7, 47–50). In addition, our studies revealed the importance of utilizing fresh tissue sections in immunohistochemical evaluation of PCX.

Although nonreceptive endometrium has been believed to be a major contributing factor to repeated implantation failure (RIF), the concept of RIF itself has lately been debated unfavorably (51–53). It is argued that RIF is the result of failure to synchronize the developing embryo with a receptive endometrium, rather than caused by an intrinsic etiology within the endometrium. In our current study, the proportions of endometrium with a PCX-positive LE did not differ between patients who had fewer and those who had more than three prior failed cycles, suggesting that nonreceptivity indicated by LE retention of PCX is not particularly associated with RIF. This observation is consistent with a recent study using ERA indicating that the prevalence of nonreceptive endometrium is similar between women who had one and those who had more prior failed embryo transfers (54).

Future studies are needed to further investigate the molecular regulation of PCX in the human endometrium for receptivity. In addition, prospective studies and randomized trials are needed to further validate the clinical applications of PCX in evaluating endometrial receptivity.

In conclusion, endometrial epithelial PCX inhibits implantation of human embryos, and luminal epithelial PCX in the putative receptive endometrium is associated with implantation failure in IVF patients. Thus, downregulation of PCX in LE is essential for endometrial receptivity for successful embryo implantation. In addition, our study suggests the potential utility of PCX in aiding the evaluation and optimization of the endometrium in fertility treatment.

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La podocalixina inhibe la implantación in vitro del embrión humano y la podocalixina luminal en el endometrio receptivo putativo se asocia con el fallo de implantación en el tratamiento de fertilidad.

Objetivo: Estudiar si la podocalixina epitelial endometrial (PCX) inhibe la implantación de embriones humanos in vitro en pacientes de fecundación in vitro (FIV).

Diseño: Recientemente hemos identificado la PCX como un regulador negativo clave de la receptividad del epitelio endometrial. La podocalixina se expresa en todas las células epiteliales del endometrio no receptivo, pero se regula selectivamente en el epitelio luminal (EL) para la receptividad. En el presente estudio, investigamos en primer lugar si los niveles elevados de PCX en la monocapa de cultivo de células Ishikawa inhiben la fijación y/o penetración de blastocitos humanos en modelos in vitro. A continuación, examinamos la PCX mediante inmunohistoquímica en tejidos endometriales receptivos putativos biopsiados de 81 pacientes de FIV que se sometieron a la transferencia de embriones congelados en el siguiente ciclo natural y analizamos retrospectivamente la asociación entre la tinción de PCX en el EL y el embarazo clínico como indicador de implantación exitosa.

Entorno: Universidad RMIT, Australia; Vrije Universiteit Brussel, Bélgica.

Paciente(s): Pacientes de fecundación in vitro sometidos a transferencia de embriones congelados/descongelados.

Intervención(es): N/A.

Medida(s) principal(es) de resultado: La PCX epitelial endometrial inhibe la implantación de embriones humanos in vitro y en pacientes de FIV.

Resultado(s): Niveles elevados de PCX en la monocapa de cultivo de células Ishikawa inhibieron significativamente la fijación y la penetración del blastocisto. Entre los 81 tejidos putativos receptivos, el 73% fueron negativos, pero el 27% fueron heterogéneamente positivos para PCX en EL. La tasa de embarazo clínico fue del 53% en aquellos con un LE negativo para PCX, pero sólo del 18% en aquellos con un EL positivo para PCX. Si el EL era positivo para PCX, la probabilidad de no tener un embarazo clínico fue del 4,95 (95% de confianza, 1,48-14,63).

Conclusión (es): Podocalyxin inhibe la implantación de embriones. La evaluación de PCX puede ayudar a evaluar y optimizar la receptividad endometrial en el tratamiento de fertilidad.